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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Each year, more than 34 million musculoskeletal injuries or organ repair or replacement surgeries, and over 300,000 cases of peripheral nerve injuries resulting in upper extremity paralytic syndrome, are reported in the U.S. alone. Active duty military personnel are more prone to orthopedic injuries involving large extremity nerve injuries than civilians. The objective of the present proposal is to develop a novel structured nanofibrous biodegradable nerve graft system that present ECM protein, neurotrophic factor, and pre-seeded with bone marrow stromal cells in rotating bioreactors for enhancing peripheral nerve regeneration to the level comparable to autograft. We determined a 0.1 mm wall thickness to provide a greater surface area and favorable environment for nerve regeneration compared to a 0.2 mm wall thickness based on characterization testing and in vitro studies. We have successfully incorporated PEG, a cross linking agent, into the inner aligned fibers of the nerve graft in the past. We observed that the 2mg Laminin PCL/PEG and 4mg Laminin PCL/PEG groups yielded the highest neurite extension and density. In our future work, we will incorporate the optimal concentration of growth factors and bone marrow stromal cells in our scaffolds and ultimately assess the outcome through animal studies.

15. SUBJECT TERMS

Tissue engineering, Nerve regeneration, Extracellular matrix, Growth factors, Bone marrow stromal cells, Bioreactor, Nanofibers

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1. **INTRODUCTION:** Tissue loss or organ failure is one of the most devastating and costly problems in health care.[1] Each year, more than 34 million musculoskeletal injuries or organ repair or replacement surgeries, and over 300,000 cases of peripheral nerve injuries resulting in upper extremity paralytic syndrome, are reported in the U.S. alone. The number of orthopaedic procedures to treat various ailments has increased in recent years as baby boomers age and overseas US military exercises result in soldier causalities.[2] Active duty military personnel are more prone to orthopedic injuries involving large extremity nerve injuries than civilians.[2] Current treatment options for patients include tissue transplantation of autografts or allografts and utilization of synthetic replacements composed of polymers. However, each strategy suffers from a number of limitations. For example, autografts and allografts are often associated with the limited availability and risks of immunogenicity, respectively. Due to the inherent limitations of using autografts, we propose to use the tissue engineering approach to design a novel nerve guidance conduit. The objective of the present proposal is to develop a novel structured nanofibrous biodegradable nerve graft system that present ECM protein, neurotrophic factor, and pre-seeded with bone marrow stromal cells in rotating bioreactors for enhancing peripheral nerve regeneration to the level comparable to autograft. We hypothesize that the proposed novel structured nanofibrous biodegradable grafts will provide the micro environment, bioactivity, transport features and mechanics ideal for enhancing the rate of nerve regeneration and healing critical sized nerve defects. We further hypothesize that the grafts seeded with bone marrow stromal cells will accelerate the tissue regeneration, resulting in formed tissue possessing the biochemical composition and mechanical properties of native tissue. Based on the encouraging findings from our previous studies, we propose to create an ECM mimetic, NGF functionalized bioactive graft. We further propose cell therapies based on seeding bone marrow stromal cells (MSCs) on the scaffolds in bioreactors prior to implantation in rat critical sized nerve defect model.

2. **KEYWORDS:** nerve regeneration, nerve guidance conduit, laminin, polycaprolactone, ECM, bone marrow stromal cells

3. **OVERALL PROJECT SUMMARY:**

   In the annual period from September, 2013 to September 2014, we have successfully completed all the milestones in the proposed SOW for this period of time. We have successfully designed, fabricated and characterized the novel structured nanofibrous nerve conduits. We have characterized the morphology, porosity, degradation and mechanical properties of the scaffold. We assessed neurite extension and neurite density of PC12 cells on our novel structured scaffolds. We incorporated extracellular matrix proteins into our scaffolds to assess optimal concentration, its stability, and bioactivity. We incorporated nerve growth factors into our scaffolds to improve their bioactivity and assess the optimal concentration to incorporate and its stability. Polymeric blends and composite approach was adopted to improve the mechanical properties, degradation behavior and cell interaction in the scaffold design. Tensile tests and initial cell compatibility was also evaluated. Specifically, we have completed the following:

   - Fabricated nerve grafts as we had proposed with defined wall thicknesses, different distributions of inner aligned nanofibers, different diameters of inner aligned nanofibers, and concentrations of outer nanofibers.

   We characterized the nerve grafts via morphology, porosity, degradation, and mechanical testing. (0-3 months)
   - Morphology: The wall thickness, gap width, and porosity dictate the nerve graft properties. We initially aimed to design spiral-structured nerve grafts with a gap width of 200μm that will have two different wall thicknesses namely 0.2mm and 0.4mm. However based on thorough assessment we decided to develop spiral-structured nerve grafts with a gap width of 200μm with two different wall thicknesses, 0.1mm and 0.2mm due to the fact 0.4mm had greater thickness than desired for an optimal nerve graft (Figure 1; Figure 2). Different wall thickness was achieved by using

different salt sizes. The 0.1mm wall thickness nerve graft was developed with 5ml of 8% PCL solution applied over 100µm salt, whereas the 0.2mm wall thickness was developed with 6ml of 8% PCL solution applied over 200µm salt. The salt size and volume of PCL solution further impacted the porosity.
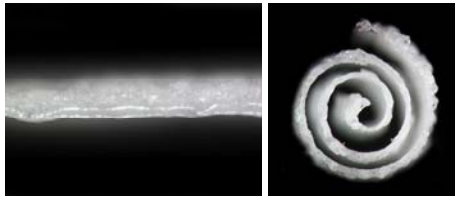

Figure 1. Stereomicroscopic images of the nerve conduit with thickness of the 0.1mm wall.
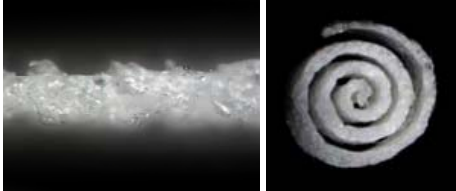

Figures 2. Stereomicroscopic images of the nerve conduit with thickness of the 0.2mm wall.

Figure 3 is the SEM images of the 0.1mm nerve graft morphology as well as the inner aligned fibers that were electrospun using 8% PCL/HFIP and 16% PCL/HFIP solution. Fig. 3 (left) clearly shows the surface of the nerve graft and the pores are visible. The size of the inner aligned fibers with 16% PCL/HFIP was 1090.28 ± 56.1nm (Fig. 3 right); and for the 8% PCL/HFIP the inner aligned fibers were 429.71 ± 112.07nm (Fig. 3; middle). The size of the pores was approximately 147.2223 ± 45.52975µm.

Figure 4 is cross sectional images of the 0.1mm as well as the tubular scaffolds that were fabricated for this study.
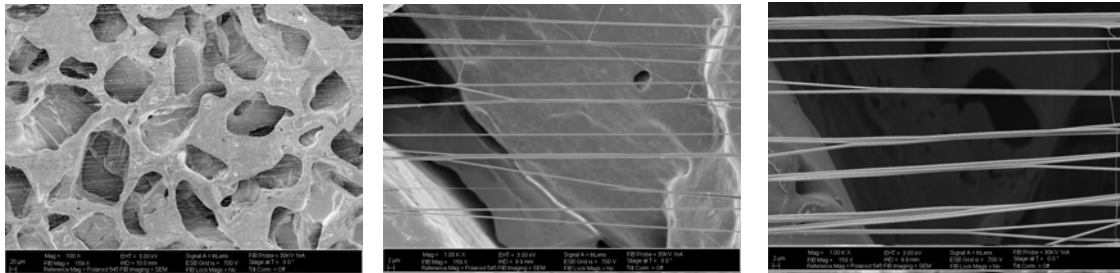
Figure 3. An SEM showing the morphology of a 0.1mm scaffold (left),
the morpohology of the inner fiber diameter for 8% (middle), the morphology of the inner diamete for 16% (right).
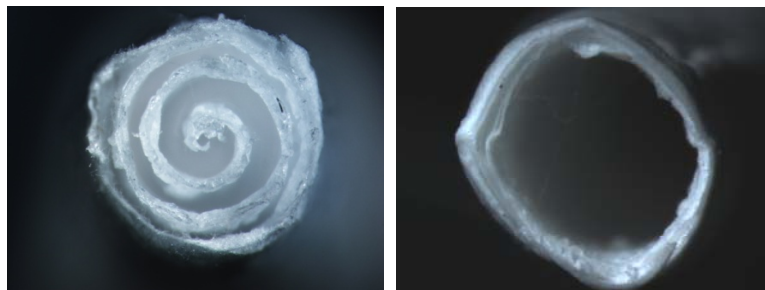
Figure 4. 0.1mm spiral cross section (left) and the tubular scaffold cross section (right).

o <u>Porosity</u>: Initially we had proposed the use of polymer weight ratios of 1:3 and 3:1 to achieve optimal pore properties and stability, however we applied salt sizes of < 100µm and 200µm-250µm to achieve scaffold thicknesses of 0.1mm and 0.2mm, respectively (Figure 5). This allowed us to successfully fabricate softer nerve grafts for this study which mimics the native nerve.
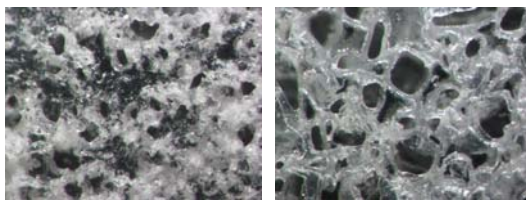
Figure 5. Stereomicroscopic images illustrating the pore size of the 100µm (left) and 200µm (right) nerve grafts respectively

We developed 4 samples for each of the 21 groups to be studied for porosity. We used the following technique to calculate porosity: we imaged the cross section of each sample of each of the 21 groups, obtained 15 measurements of the different layers seen in the cross section, averaged the measurements to yield a single thickness value, and then measured the mass of each sample. The average thickness value was used to determine the volume of the scaffolds. The mass and volume were used to determine density of the scaffold. The following equation was then used to calculate the porosity for each scaffold in each group.

$$Porosity = 1 - \frac{Density\ of\ Scaffold}{Density\ of\ Material}$$

The material in this case was Polycaprolactone (PCL). The results for porosity are shown below with images of the 0.1mm scaffold cross section and 0.2mm scaffold cross section.
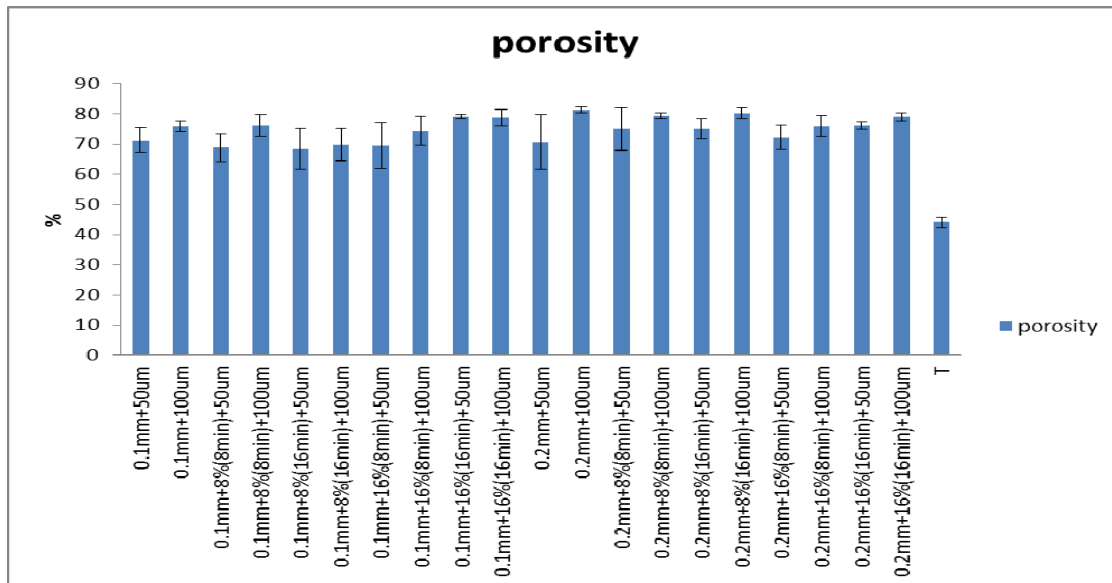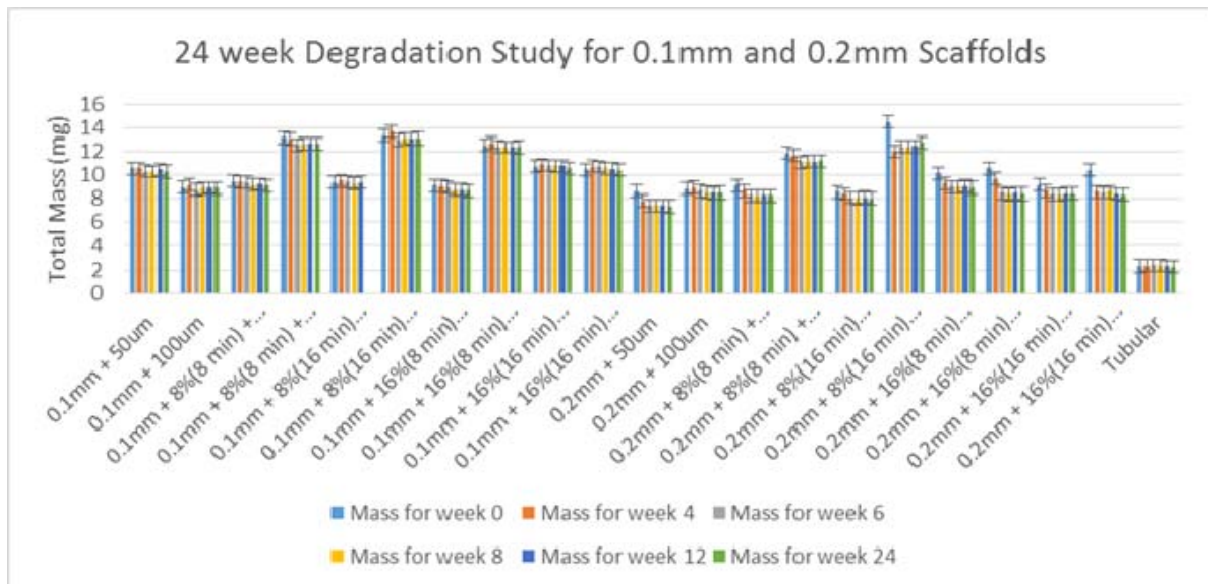

Figure 6. Porosity Study on the 0.1 mm wall thickness and 0.2 mm wall thickness groups

The porosity data in Figure 6 shows the percentage of pores with respect to each group of scaffolds. The results are approximately between 70% and 80%, in that the scaffolds are composed of pores with the exception of the tubular group. Despite the difference in wall thickness the porosity of the 0.1mm and 0.2mm groups is similar, however in comparison to the tubular conduit we have shown significant improvement. Having porous scaffolds is advantageous in nerve regeneration in that it will allow for nutrient exchange to take place to ensure healthy nerve regeneration. The porosity data above shows the percentage of pores with respect to each group of scaffolds. Having porous scaffolds is advantageous in nerve regeneration as they allow for nutrient exchange to take place to ensure healthy nerve regeneration. Approximately between 70% and 80%, in that the scaffolds are composed of pores with the exception of the tubular group. In comparison to the tubular scaffold we have shown significant improvement.

o   Incorporate nanofibers onto scaffolds: Nanofiber matrix coating thickness and fiber diameter influence scaffold properties in terms of surface area, porosity, and mechanical properties. We

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incorporated two different nanofiber diameters: 80nm (nano range), and 800nm (submicron). Initially we had proposed two different fiber matrix thickness: 2µm and 5µm in our design, however based on thorough assessment we decided to pursue fiber matrix with 50% coverage and 100% coverage which were achieved based on a variation in time dedicated to electrospinning, 8 minutes for the former and 16 minutes for the latter.

o   Outer tubular matrix: Two types of outer tubular matrix were electrospun onto the aforementioned nerve graft types, 50µm and 100µm.

o   Nerve Graft Types: In this design, the parameters that defined the properties of the inner spiral scaffolds are the height, diameter, wall thickness, gap width, salt particle size, fiber diameter, fiber distribution, and fiber mat thickness. Thus we fabricated 21 different scaffold compositions and will characterize them for morphology and porosity analysis (n=4), mechanical testing (n=6), and degradation (n=4) study to achieve statistical significance.

o   Degradation Testing: For each of the 21 groups we fabricated 4 samples for degradation testing. We performed the degradation using the following technique: we obtained the dry weight of each sample of each group, then placed each sample in 1 mL of phosphate buffered saline (PBS) until each time point indicated in our proposal (4, 6, 8, 12, 24 weeks), 2 days prior to reaching each time point we removed all of the PBS and allowed each sample to dry, and finally we measured the mass at each time-point. Following the measurement of the mass we placed each sample in 1 mL of PBS again and repeated the procedure at each time-point. The data up to time-point of 24 weeks is shown below.

Figure 7. Degradation Study on the 0.1 mm wall thickness and 0.2 mm wall thickness groups

The degradation data in Figure 7 shows the total mass of the scaffolds at week 0 (the initial dry weight), week 4, week 6, week 8, week 12, and week 24. Degradation is a time dependent mechanism and the optimal scaffold is one that degrades as soon as the nerve has regenerated. While the mass may be influenced by the presence of inner aligned fibers and outer fibers, it did not cause a significant difference compared to other groups. As seen above the total mass of the scaffolds at each time point is similar despite the difference in wall thickness. Within a short period of time the scaffolds retained their initial mass, which indicates that PCL scaffolds degrade at a slow rate which is essential since nerve regeneration of a critical gap length (15mm) may take up to and possibly over 1 year for complete recovery. Shown above is the complete degradation study for the 24 week period as we had proposed. Along with our experimental groups we performed the

degradation study on a tubular group as a negative control. As we had stated in our previous report(s), degradation is a time dependent mechanism and the optimal scaffold degrades as soon the nerve is fully regenerated. The variation in the outer fibers, inner aligned fibers, and thickness of the scaffold(s) did not result in a significant difference in initial mass and mass change. While none of the above scaffolds did not fully degrade, this is expected in the use of PCL and is useful in treating peripheral nerve injuries as critical gap length can take up to and sometimes over a year to fully recover. All of the scaffolds between showed some mass loss from their initial dry weight to their final endpoint of 24 weeks.

- o Mechanical Testing: For each of the 21 groups 6 samples were fabricated for a total of 126 samples. Composite spiral structured and tubular nerve grafts were tested using Instron mechanical testing instrument. The specimen test length was 20mm with wall thickness of 0.24mm and extended at the rate of 5mm/min.

  Spiral PCL scaffolds of 0.1mm thick PCL sheet exhibited Young's Modulus of 50-77MPa and tensile load of 3.5-7N. Nerve grafts fabricated from 0.2mm thick PCL sheets showed significantly decreased Young's modulus and tensile load of 10-20MPa and 1.8-3N respectively. The properties of the PCL sheet and the composition of the fibers modulated the mechanical properties of the spiral structures scaffolds. From Group 1, which is composed of PCL sheet of 0.1mm thickness, graft designs C5 and C6 (0.1mm PCL + 8% Aligned Fibers (AF) (100% coverage) + 50 or 100um Outer Fibers (OF)) had the highest modulus as well as the highest tensile strength of those tested. Grafts fabricated from 0.2mm thick PCL sheet did not exhibit significant differences among the various fiber compositions; all grafts incorporating fibers had much improved properties as compared to PCL sheet alone. Table 1 presents modulus (MPa) of the various graft designs (n=6). Table 2 presents the tensile load (N) of the various graft designs having tubular wall thickness of 0.24mm (n=6).

Table 1	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	
Modulus (MPa)	54.06	53.21	50.83	62.33	77.12	75.84	48.17	52.88	46.94	58.04	
	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C23
Modulus (MP3a)	13.92	18.71	16.59	19.33	16.88	18.05	17.78	16.48	19.94	10.23	10.14

Table 2	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	
Tensile Load (N)	3.97	4.15	3.88	7.10	6.20	6.23	4.20	4.70	3.60	4.65	
	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C23
Tensile Load (N)	1.95	3.04	2.70	2.86	2.70	2.98	2.22	2.08	2.78	1.80	2.1

- We assessed neurite extension and neurite density of PC12 cells on our novel structured scaffolds. In Vitro Cell Study: Shown below in Fig. 8a-8f are the results of the PC12 neurite extension.
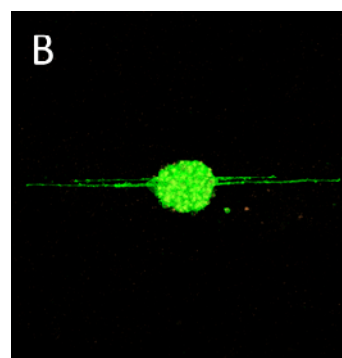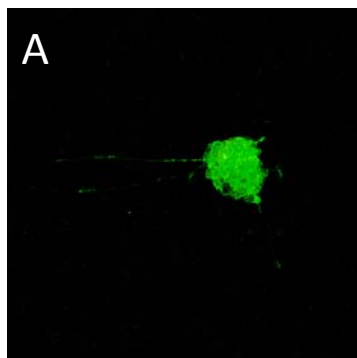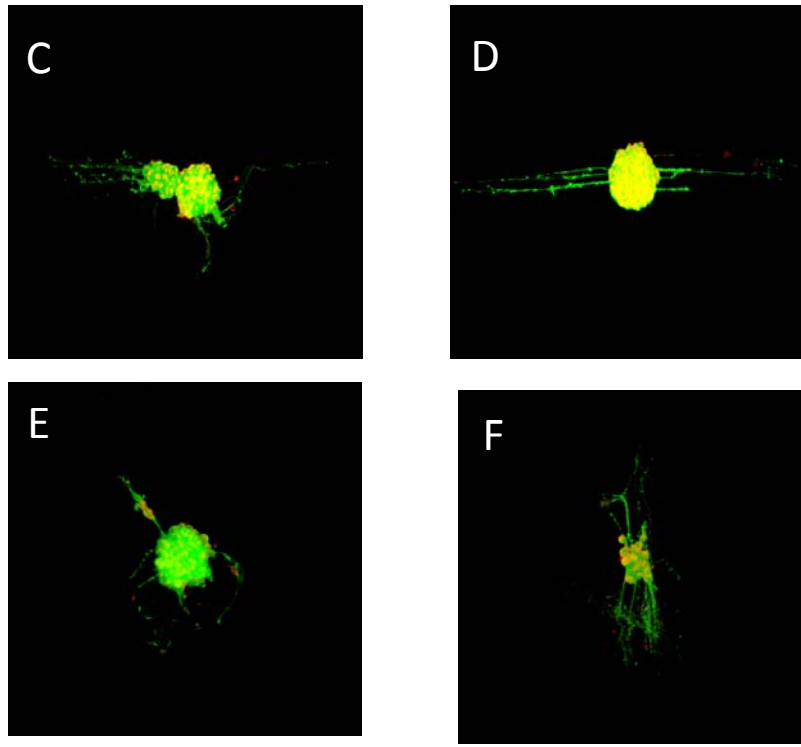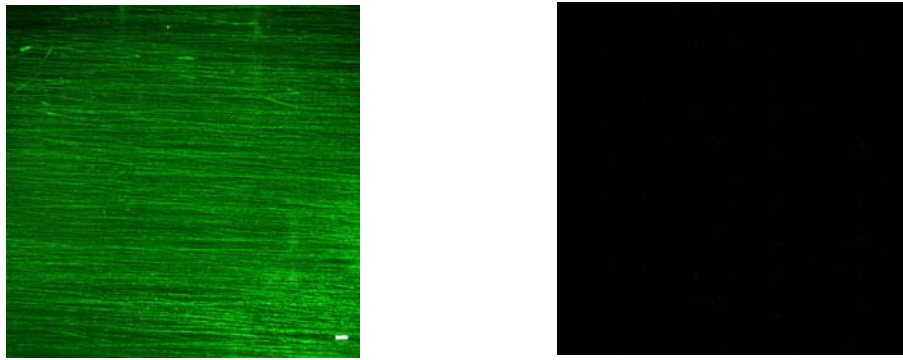
Figure 8. PC12 neurite extension on (A) 0.1mm + 8%(8 min), (B) 0.1mm + 8%(16 min), (C) 0.1mm + 16% (8 min), (D) 0.1mm + 16%(16 min), (E) 0.1mm, and (F) tubular.

We cultured PC12 cells on 0.1mm scaffolds with variations of inner aligned fiber distribution and the concentration of PCL/HFIP solution we used to electrospin the aligned fibers. We used 0.1mm thickness scaffolds as they showed better Schwann cell attachment than 0.2mm thick scaffolds. As can be seen above the neurite of each experimental group with inner aligned fibers was better than the tubular group and the 0.1mm group. The inner aligned fibers allowed showed better neurite extension as they provide guidance and direction to extending neurite(s).

Compared to the 0.1mm + 8% (8 min) and 0.1mm + 16% (8 min), the 0.1mm + 8% (16 min) and 0.1mm + 16% (16 min) had a better presence of neurite extension. Based on these images we can assume the amount of inner aligned fibers present influences neurite extension. Inner aligned fibers have shown to influence neurite extension in literature and our study has shown that the amount of inner aligned fibers can significantly influence neurite extension.

The 0.1mm + 8% (16 min) and 0.1mm + 16% (16min) had a greater amount of fibers present than the 0.1mm + 8% (8 min) and 0.1mm + 16% (8 min). A greater amount of fibers covers a greater area of the scaffold so there is more potential to guide more neurites which is why the 0.1mm + 8% (16 min) and 0.1mm + 16% (16min) groups showed better neurite extension. The 0.1mm + 8% (8 min) and 0.1mm + 16% (8 min) inner fiber coverage parallels the behavior of the 0.1mm group and the tubular group as there is a significant area of the scaffold without fibers which limits the amount of neurite that can be guided and in turn may influence how many neurite can extend from the PC12 cells.

Figure 9. PCL with inner aligned fibers blended with Poly(ethylene glycol) bis(amine) then stained with FITC to indicate the distribution of (left) PEG on the inner aligned fibers and (right) PCL with inner aligned fibers without PEG.

As seen in Fig 9 (left) we blended PCL with poly(ethylene glycol) bis(amine) (PEG) and stained it with FITC but Fig 9 (right) shows the absence of PEG with FITC staining. PEG is a cross linking agent that allows for surface modification of so we can later incorporate ECM proteins. We have successfully incorporated PEG into the inner aligned fibers of the nerve graft and having the uniform distribution as seen in Fig 9 (left) allows the cross linking of an ECM protein. Without the presence of a cross linking agent an ECM protein can desorb from the surface or have uneven coating once implanted however by retaining proteins within inner aligned fibers allows proteins to serve as contact guidance cues.

• We incorporated extracellular matrix proteins into our scaffolds to assess optimal concentration, its stability, and bioactivity.
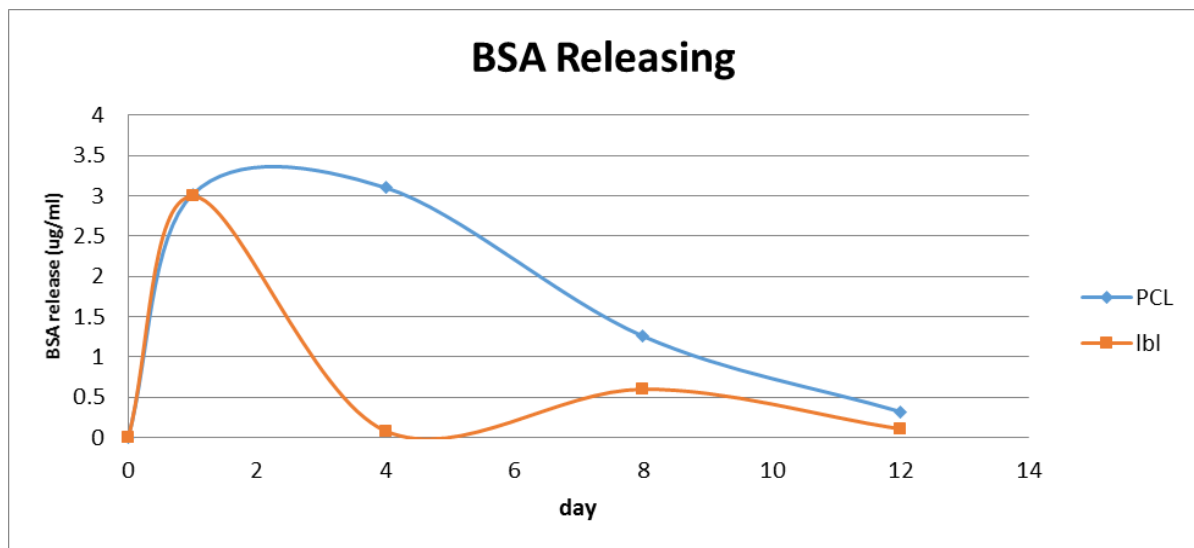

**Figure 10.** BSA release for a 12 day period using a layer-by-layer and a standard PCL approach.

In our work we used bovine serum albumin (BSA) to assess protein release techniques. BSA is the standard model used to assess controlled release of a drug or protein. We compared a layer-by-layer (LBL) approach to the standard approach, which is injecting the desired concentration of the protein and drying it. We performed a protein loading efficiency of each technique as well as the BSA release for a 12 day period. It is well known that in the absence of controlled release the concentration of the protein releases in non-uniform amounts and can be potentially desorbed within the first few days of implantation. Our layer by layer approach, which involved modifying the surface charge of PCL by placing the nerve grafts in polyethylenimine (PEI) solution overnight so PEI became the surface layer. The scaffolds were then treated with Tannic Acid/BSA, water, chitosan, and water. We used 30 µg of BSA in 50 µL of PBS for loading into each scaffold.

As seen from Fig. 10 the BSA had a more controlled release with the LBL approach compared to the standard approach with PCL nerve grafts. The loading efficiency, which measured the amount of protein a nerve graft can retain, was better for the layer by layer approach as opposed to the standard PCL approach. The PCL had a 45.3% retention rate whereas the LBL had a 66.5% retention rate.

Shown below in Figs. 11 and 12 are the Bicinchoninic Acid Protein Assay (BCA) standard curve for Laminin.
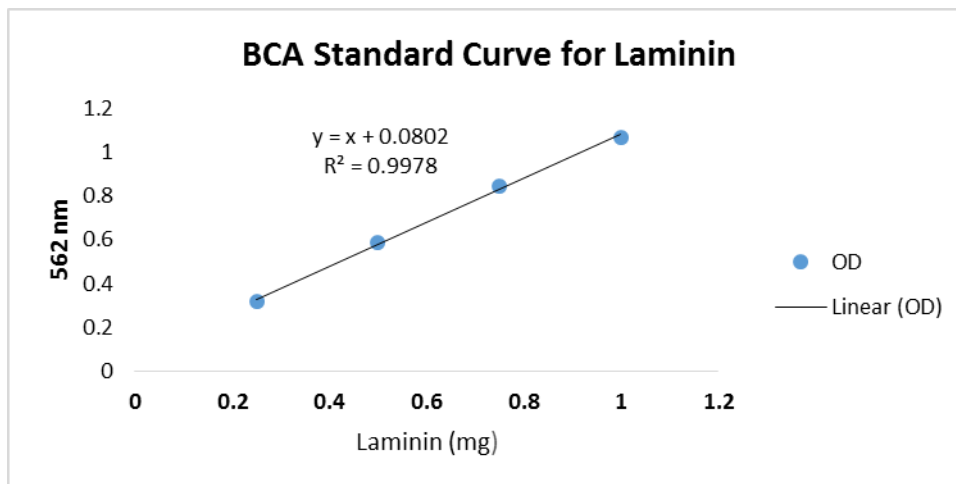
Figure 11. BCA standard curve for Laminin from 0.2 mg to 1 mg at 562 nm
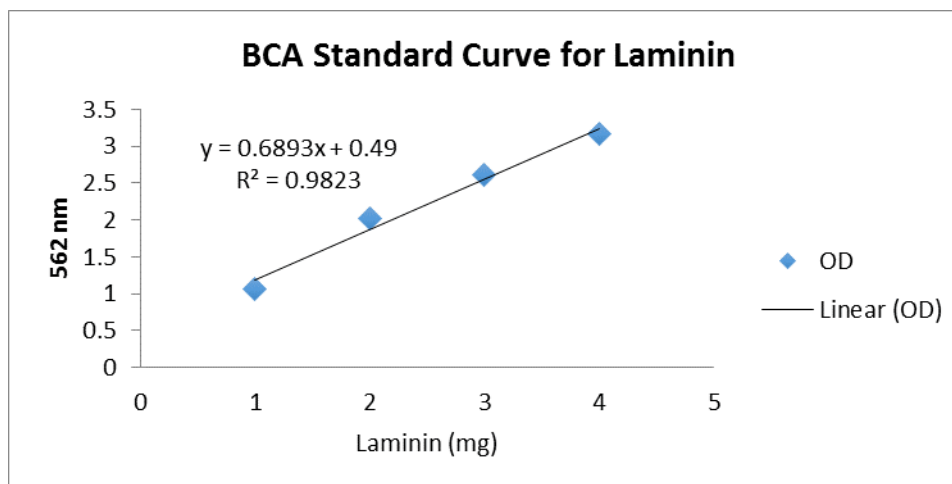
Figure 12. BCA standard curve for Laminin from 1 mg to 4 mg at 562 nm

BCA is a widely applied assay for protein determination. It is applicable for a broad range of protein concentrations. A wavelength of 562 nm is applied as this assay exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. Different concentrations of Laminin for the standard curves shown Figs. 11 and 12. Having a standard curve for different ranges of concentrations allows us the opportunity to assess the concentration of protein for measured absorbance of unknown samples.

Table 3. Laminin Attachment based on Concentration of Crosslinking

	PCL	0.5 mg Laminin PCL/PEG	2 mg Laminin PCL/PEG	4 mg Laminin PCL/PEG
Laminin (mg)	0.002866333	0.040533	0.173025291	0.460176508

PEG is a cross linking agent that allows for surface modification of so we can incorporate ECM proteins. We have successfully incorporated PEG into the inner aligned nanofibers of the nerve graft in the past. In the absence of a cross linking agent, an ECM protein can desorb from the surface once implanted however retaining proteins within inner aligned fibers lets proteins act as contact guidance cues. Table 3 indicates the amount of Laminin that was cross-linked onto the aligned nanofibers. The results showed that as the protein concentration increased, more Laminin was attached.

PCL

0.5 mg Laminin PCL/PEG

2 mg Laminin PCL/PEG
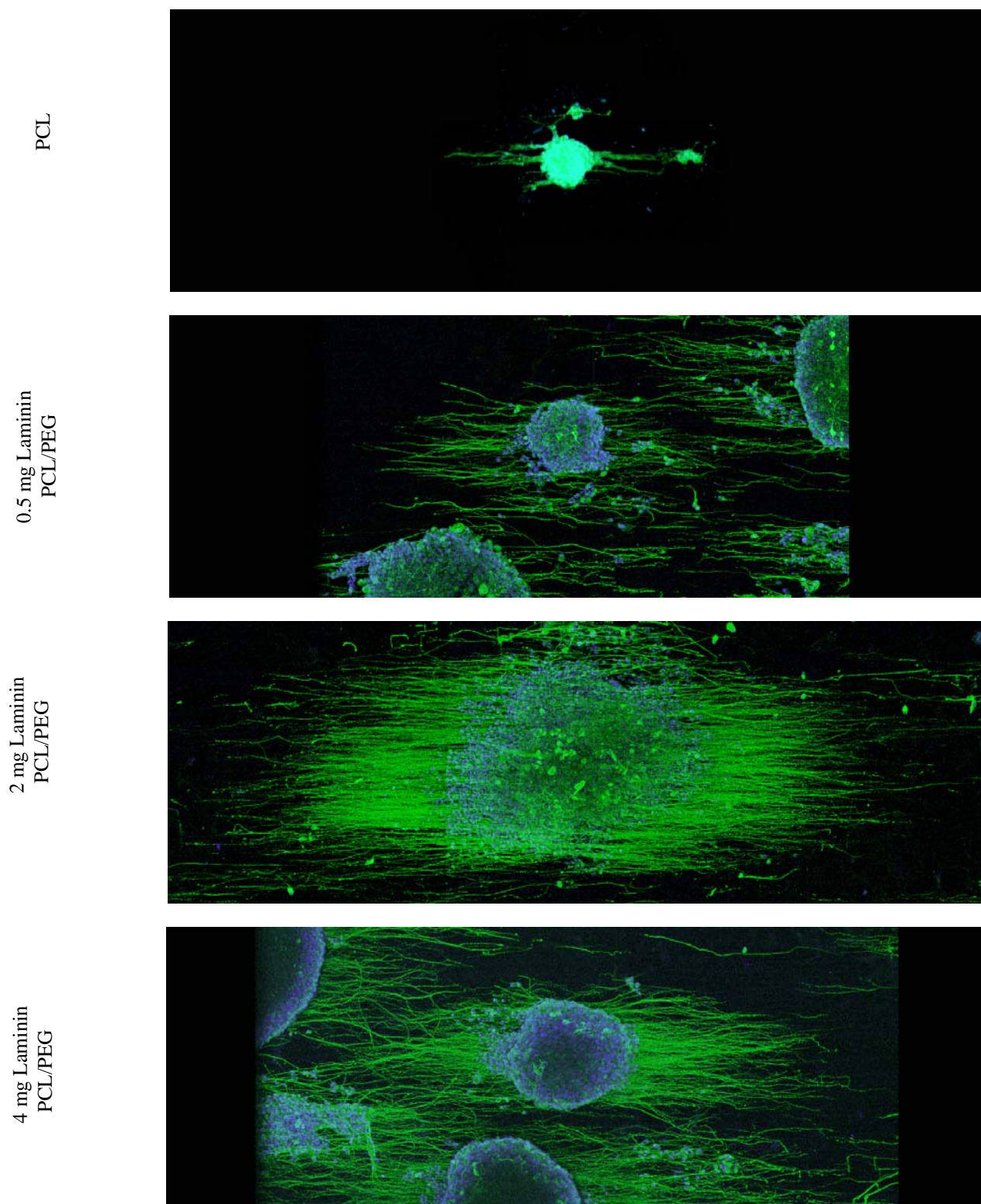
4 mg Laminin PCL/PEG

Figure 13. PC12 neurite elongation on PCL nanofibers (A), PCL-PEG nanofibers with 0.5 mg Laminin (B), PCL-PEG nanofibers with 2 mg Laminin (C), and PCL-PEG nanofibers with 4 mg Laminin (D).

Once we characterized the amount of Laminin that was cross-linked for each concentration of groups, we seeded PC12 cells on the scaffolds. Using confocal microscopy and FITC staining we assessed PC12 cell neurite extension and neurite density. From Fig. 13 (A-D) images, we can tell that the laminin coated groups had a significant effect on PC12 differentiation. As mentioned previous quarterly reports, the aligned nanofibers density influences neurite extension and in turn neurite density. As such we used 100% nanofibers distribution and observed that the 2mg Laminin PCL/PEG and 4mg Laminin PCL/PEG groups yielded the highest neurite extension and density.
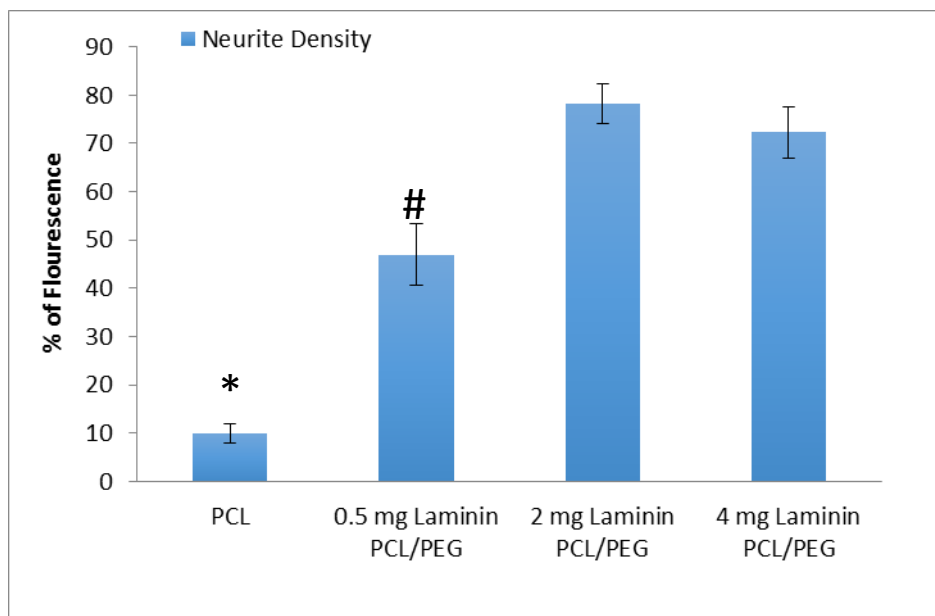
Figure 14. PC12 Neurite Density. * and # indicate P < 0.05
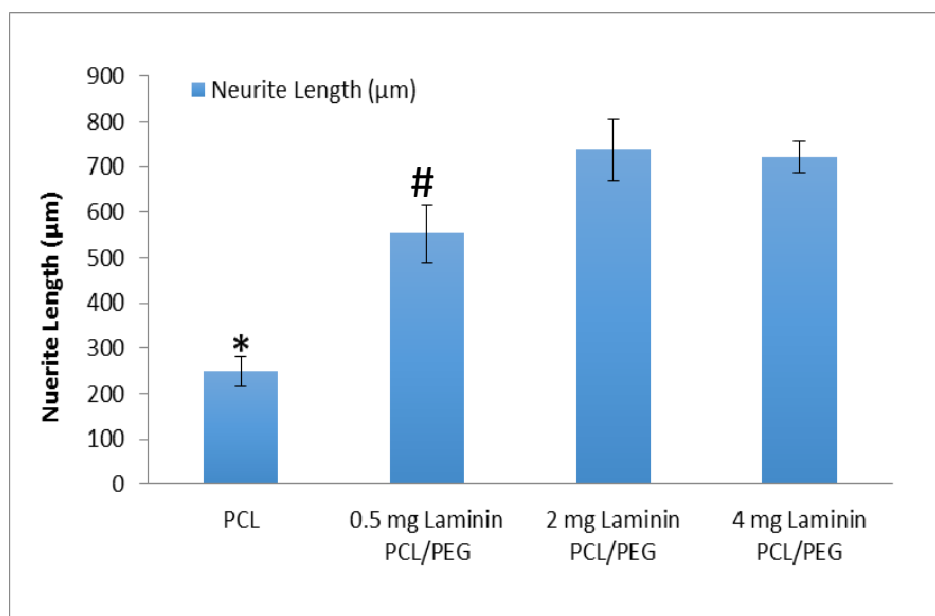
Figure 15. PC12 Neurite Length. * and # indicate P < 0.05

We characterized the response of the PC12 cells on the scaffolds based on neurite density and neurite length (Figure 14; Figure15). As evident from these graphs the 2 mg Laminin PCL/PEG and 4 mg Laminin PCL/PEG provided the most favorable surface for the PC12 cells. * and # indicate P < 0.05

- We incorporated nerve growth factors into our scaffolds to improve their bioactivity and assess the optimal concentration to incorporate and its stability.
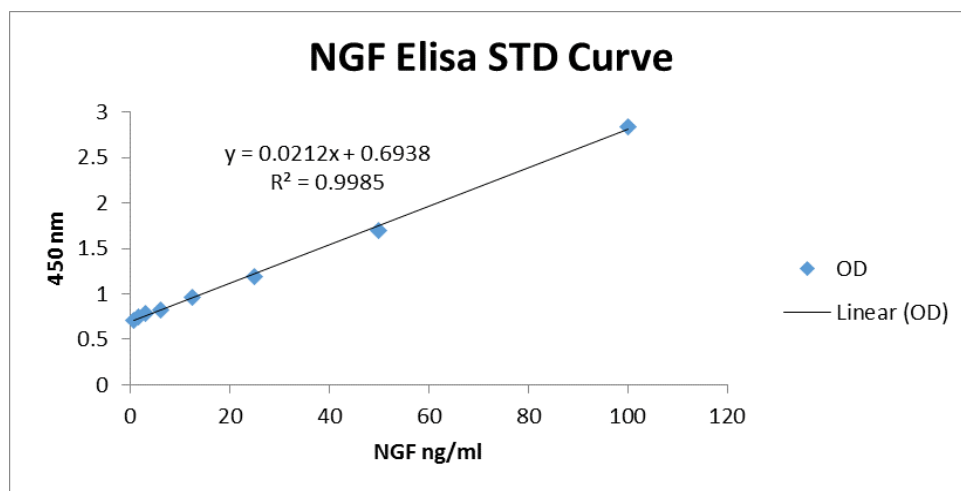
Figure 16. Standard curve for NGF from 0 ng/ml to 100 ng/ml at 450 nm

The NGF Elisa standard curve was successfully made at 450 nm for NGF concentrations between 0 ng/ml to 100 ng/ml (Figure 16). By having a standard curve we will be able to assess concentrations of NGF for unknown samples with measured absorbance values. For the future milestones we will move on to the next study which will be NGF release from nanofibers. The releasing profile and in vitro will be studied simultaneously.

Moreover, composite scaffold PCL nanofiber matrices with Na-Alg coating and blending approach for PCL- gelatin, and electrically conducting polymers were investigated for improving various properties for nerve conduits.

Composite Scaffold-PCL Nanofiber Matrices (random and aligned) with and without Na-Alg coating: It was hypothesized that the composite scaffolds derived from nanofiber matrices in combination with Na-Alg may able to provide right combination of mechanical properties, bioactivity and cell attachment features. Studies were designed to evaluate the effect of Na-Alg molecular weight on these properties. Na-Alg was treated with hydrogen peroxide to break long polymer chains into shorter fragments that increased with treatment time. Efforts were to identify a lower molecular weight Na-Alg that will provide the optimal mechanical and bioactive properties to promote BMSCs growth and differentiation. Hydrogen peroxide has been previously shown to cleave β-glycosidic bonds of Na-Alg backbone resulting in low molecular weight sodium alginates. The molecular weight of resulting sodium alginates could be controlled by varying hydrogen peroxide concentration, temperature or reaction time. The degradation studies clearly showed decrease in viscosity with respect to increase in time (viscosity for 0.5min   1hr   2.5hr   native sodium alginate). We employed 5% hydrogen peroxide and 37°C to produce Na-Alg with different molecular weights/viscosity. The PCL nanofiber matrices were incubated in native as well as Na-Alg of varying molecular weight solutions for 15 min and cross-linked with 1% (wt/v) aqueous calcium chloride solution for 15 min. PCL nanofiber matrices and native Na-Alg coated matrices served as a controls. The mechanical tests were performed as such cross-linked fiber matrices immediately after fabrication. In another study dried scaffolds were incubated in physiological conditions (pH 7.4 and 37ºC) overnight and tested for mechanical features to see the possible differences.

Blending approach-PCL-Gelatin: In an effort to improve PCL hydrophilicity and impart scaffolds with much desired bioactivity we made an effort to combine with a natural polymer gelatin. In our recent publication we have shown a novel methodology and feasibility of electrospinning chitosan alone or in combination with synthetic polymers through nitrobenzaldehyde modification (*Polym. Adv. Technol.,* 2014, 25: 507–515.). Often it is difficult to combine a water soluble polymer into an organic solvent soluble polymer at higher concentrations during electrospinning.

Nitrobenzaldehyde modification of the gelatin allowed the PCL-gelatin electrospinning. We produced nanofiber matrices using a PCL-modified gelatin at a ratio of 50:50 in a common organic solvent. Thus fabricated nanofiber matrices were cross-linked with glutaraldehyde and cross-linked fiber matrices were exposed to UV-light to remove the photoactivatable-caged compound nitrobenzaldehyde. Thus fabricated blend nanofiber matrices were subjected for mechanical properties, degradation behavior and cytocompatibility.
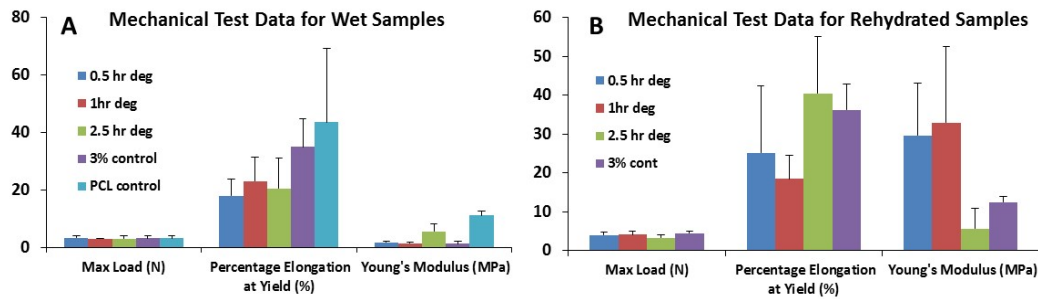
Tensile Properties:

PCL-Na-Alg Composite Nanofiber Matrices: Dog bone shaped composite fiber matrices measuring 10 X 20 mm (length breadth) and a thickness of 0.24mm following Na-Alg coating (varying molecular weight) were tested under extension at a rate of 5mm/min until failure. From the stress-strain curves different parameters such as modulus, max load and percent elongation were calculated and presented in figure 17A-D. Composite scaffolds produced with lower molecular weight (or low viscosity) Na-Alg produced similar max load as that of native Na-Alg (high viscosity). Further no significant differences were found between the samples tested immediately following its fabrication as well as after rehydrating it in a physiological environment overnight. These finding suggest that dry fiber matrices can be stored in a sterile condition and can be rehydrated prior to its use without losing its mechanical properties. It is also interesting to note that composite scaffolds obtained by coating with Na-Alg obtained by 0.5hr and 1hr hydrogen peroxide treatment showed higher modulus than the native Na-Alg coated. These findings suggest that low molecular weight Na-Alg coating is sufficient to achieve the optimal mechanical properties. Further higher erosion rate of low molecular weight Na-Alg from the scaffold system as compared to high molecular weight native Na-Alg may benefit the scaffold degradation and tissue regeneration. Comparison of tensile properties with respect to as such fabricated composite scaffolds and rehydrated scaffolds are presented in Figure 18A-B. These findings strongly support our hypothesis and degradation studies of this scaffold system are currently underway.
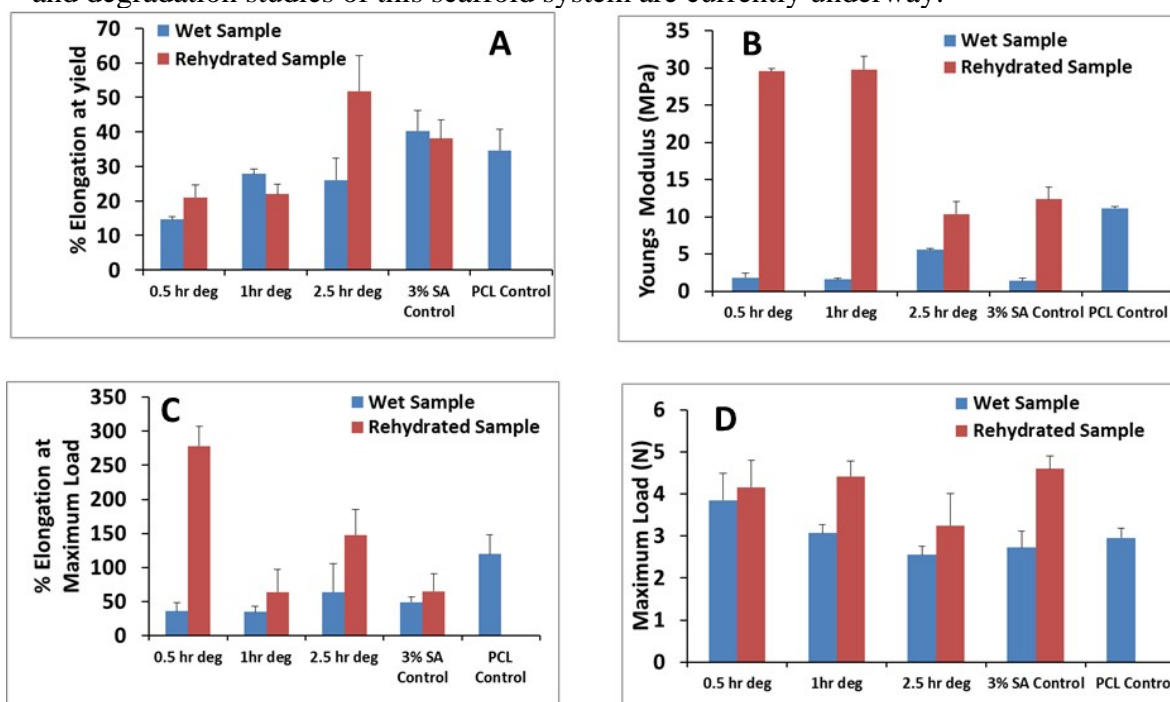
Figure 17: Tensile properties of Na-Alg coated PCL matrices where (A-B) represent % elongation at yield and Young's modulus for wet and rehydrated nerve grafts respectively. C-D represent % elongation at max load and max load matrices can take prior to failure for wet and rehydrated matrices respectively. It is evident from these figures that the rehydrated nerve grafts had improved mechanical properties over wet nerve grafts due to the combined effects of Na-Alg infiltration throughout the matrix and cross-linking

Figure 18: Effect of Na-Alg molecular weight (solution viscosity) on tensile properties of composite nanofiber matrices for (A) wet and (B) rehydrated samples. It is evident that rehydrated samples show improved max load, % elongation, and modulus over wet matrices tested immediately following fabrication. Such an improvement in tensile properties is a result of Na-Alg infiltration throughout the matrix and effective cross-linking.

### Blend Nanofiber Matrices: PCL-gelatin

Tensile properties of PCL-gelatin and PCL-modified gelatin blend nanofiber matrices are presented in Figure 19A-B. All these findings suggest that modified gelatin blends with PCL show improved mechanical properties over the neat gelatin PCL blend and PCL alone. Improved tensile properties presumably due to complete miscibility of two polymer phases in the case of modified gelatin. These findings suggest that inclusion of gelatin in PCL improve matrix hydrophilicity and cell compatibility. Matrix degradation and other properties such as hydrophilicity and pore property characterizations are currently underway.
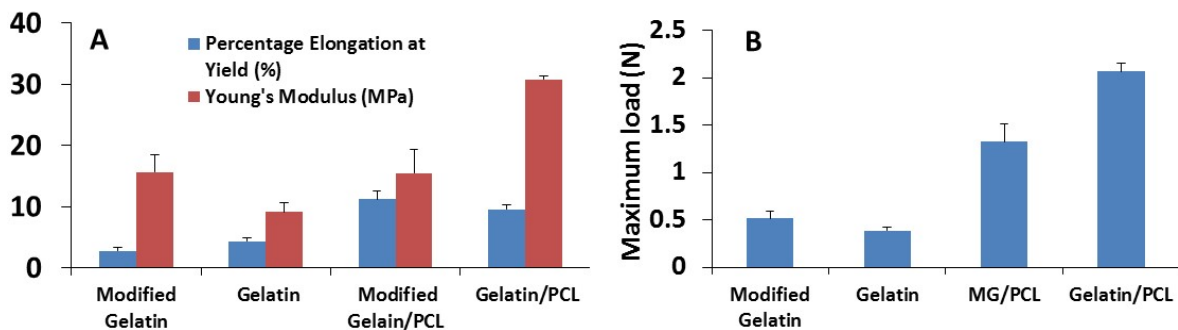
Figure 19: Mechanical properties of gelatin (modified and unmodified) and their blends with PCL. A) Percent elongation at yield and Young's modulus, the data indicates that the percent elongation at yield for modified and unmodified gelatin nerve grafts was similar, and these values were higher in case of blends of modified and modified gelatin with PCL B) Maximum load bearing capacity. The maximum load bearing capacity of the nerve grafts were increased upon modification as well as upon blending with PCL.

Preliminary Cell-Compatibility Studies:

PCL-Na-Alg composite matrices: Both aligned and randomly oriented nanofiber matrices coated with Na-Alg were seeded with human induced pluripotent stem cell (iPS) to study their ability to support cell attachment and proliferation. Following 7 day in vitro culture cell viability studies (live-dead) were performed and confocal images were taken to see neurite extensions. Figure 20A-D shows the representative confocal images for cells on composite and PCL fiber matrices. These matrices were able to support the attachment and growth of human iPS cells and neurite extensions were also evident.
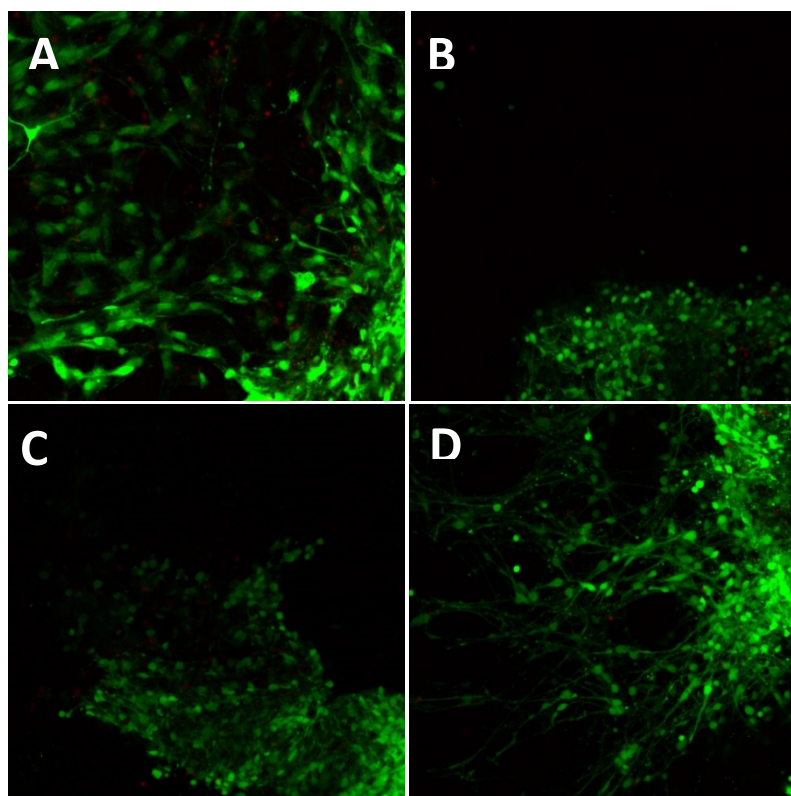
Figure 20. Live-dead assay representative confocal microscopic images presented at 10x magnification for (A) aligned, (B) random PCL, (C) composite aligned and (D) composite random fiber matrices. It is evident from the micrographs that all these matrices were able to support human iPS attachment, proliferation and neurite extensions.
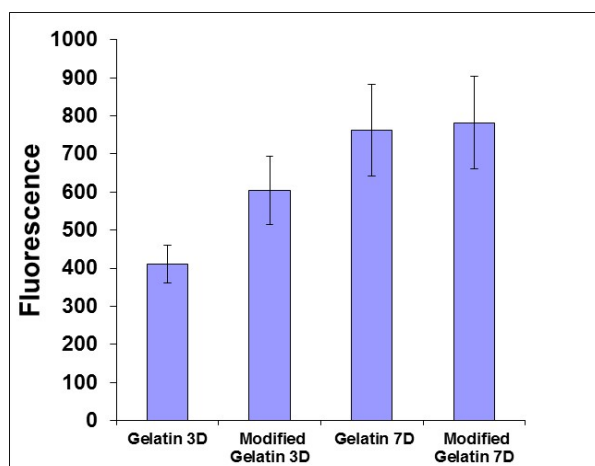
Gelatin nanofiber matrices: Nanofiber matrices containing either modified gelatin or neat gelatin were seeded with BMSCs to evaluate their ability support cell attachment, proliferation and viability *in vitro*. A representative confocal image following 3 day culture and live dead staining is presented in Figure 21 (A-B) on both the matrices. It is evident from the micrographs that these matrices were able to support the attachment and spreading of BMSCs in vitro. Cell proliferation at two different time points namely day 3 and 7 is presented in Figure 22. It is evident from the Figure 22 that both the matrices were able to support fibroblasts growth and gelatin modification looks promising both in terms of improving the mechanical properties as well as cell compatibility.
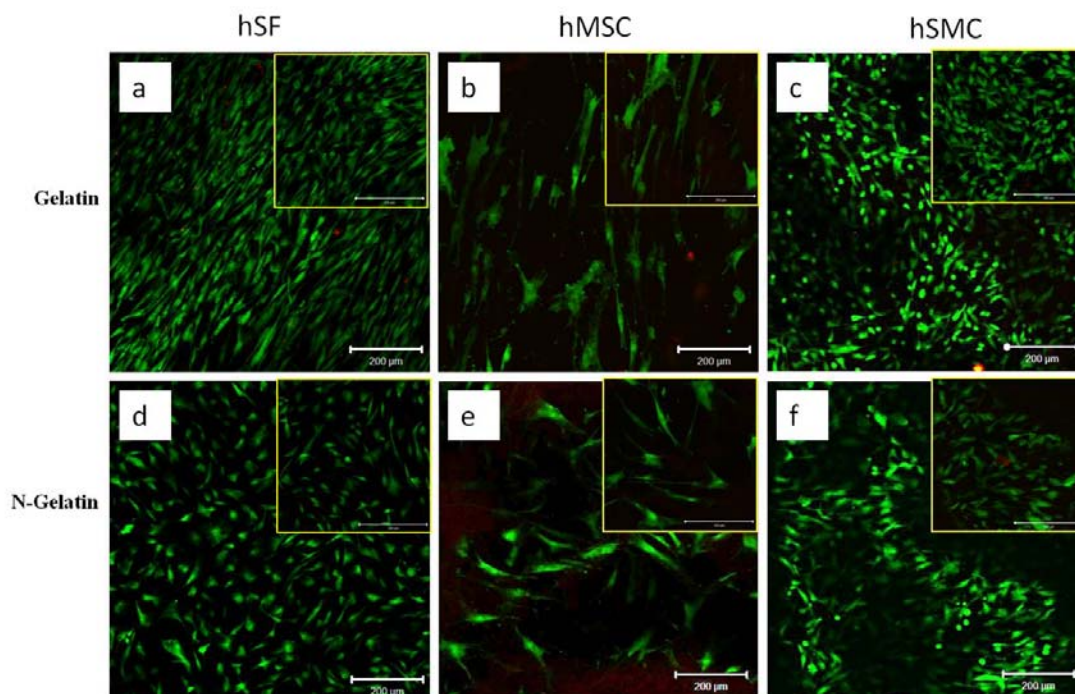
Figure 21. Cell attachment and viability was assessed using Live/Dead cell viability assay where green and red shows live and dead cells respectively. Human skin fibroblast, hSFs (a, d), human mesenchymal stem cells, hMSCs (b, e) and human skeletal muscle satellite cells, hSkMSCs (c, f) were visualized using confocal microscopy after 3 day of incubation on regenerated gelatin scaffolds. Overall cells look healthy and have proliferated well on the modified gelatin fibers. For all three cell types, the regenerated fibers show excellent biocompatibility and cell attachment with few or no dead cells. Top panel corresponds to pure gelatin fibers and bottom panel corresponds to N-Gelatin fiber matrices. Objective: 10X. Insets show 20X images for their respective conditions. Cells have attained elongated morphology and are extending along the fibers (arrow).

Figure 22. DNA assay indicating the cell proliferation over a period of 7 days on neat and modified gelatin nanofiber matrices. The progressive fibroblasts growth with respect to time time ensured the matrix compatibility with the cells.

Electrically conducting polymers for regenerative engineering applications: sulfonated ionic membranes

Conductive polymers have found extensive application in fuel cells, sensors and more recently as scaffolds for tissue and organ regeneration. Scaffolds that can transmit electrical impulses have been shown to be beneficial in regeneration of tissues like muscle and nerve that are electroactive in nature. Most cellular events and cell functions are regulated by ion movement, and their imbalance is the cause of several diseases.

We report synthesis and characterization of sulfonated polymers of poly(methyl vinyl ether-alt-maleic anhydride) (PMVEMA), poly(ether ether ketone) (PEEK), poly(ether sulfone) (PES) and poly(phenylene oxide) (PPO) and evaluate their potential for tissue regeneration. The ionic conductive property stems from the presence of sulfonic groups on the polymer backbone. The structure of the polymer was confirmed using Fourier Transform Infrared Spectroscopy and membrane hydrophicity was determined by water contact angle measurement. The electrical conductivity of these sulfonated membranes was found to be 53.55, 35.39 and 29.51 mS/cm for SPPO, SPEEK and SPMVEMA, respectively. The conductivity was directly proportional to the sulfonic acid content on the polymer backbone. The ionic membranes namely SPPO, SPEEK and SPMVEMA demonstrated superior cell adhesion properties (~7–10 fold higher) than cells seeded onto tissue culture polystyrene. The sulfonated membranes exhibited static water contact angle in the range of 70–76°. The membranes supported the proliferation of human skin fibroblasts over 14 days in culture as evidenced by confocal and electron microscopy imaging (Figure 23). The ionic materials reported in this study may serve as scaffolds for a variety of nerve regeneration, tissue healing and drug delivery applications. Results of these achievements are published in *Polym. Adv. Tech.,* 2014, DOI: 10.1002/pat.3385.
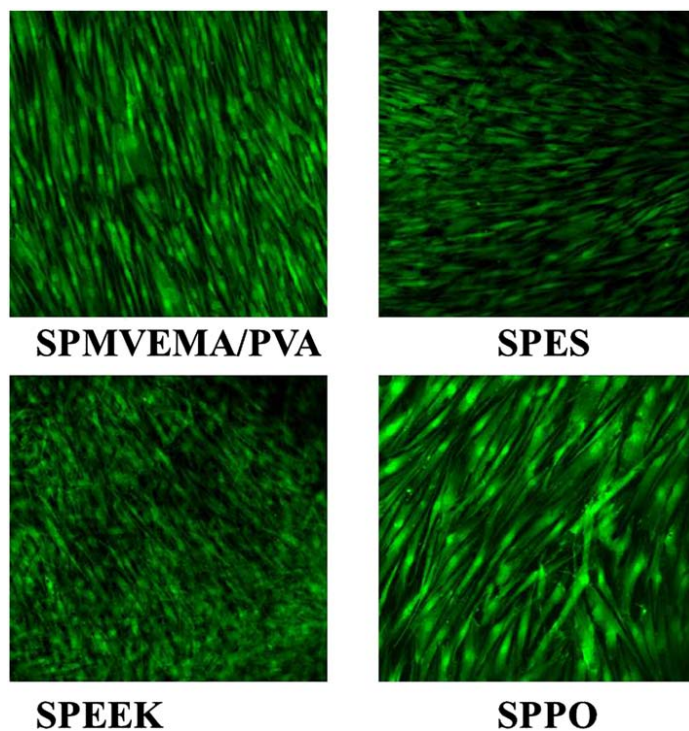
Figure 23: Confocal microscopy images of huSkF on sulfonated ionic membranes showing characteristic spindle-shaped cell morphology. The membrane exhibited high biocompatibility and supported cellular adhesion.

4. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

- We fabricated novel spiral structured scaffolds of different wall thicknesses: 0.1mm and 0.2 mm. Based on the results of our characterization methods shown above, we observed that a 0.1 mm thick scaffold yields more spiral layers and which in turn can reduce any limitation of axonal infiltration and provide a greater surface area for nerve regeneration. Additionally we observed that Schwann cell attachment was significantly higher for the 0.1mm scaffolds as opposed to the 0.2mm scaffolds.
- We assessed the impact of nanofiber distribution and nanofiber diameter on neurite extension and neurite density of PC12 cells by electrospinning 8% PCL/HFIP and 16% PCL/HFIP. The concentrations of solution impacted the diameter of the nanofibers whereas the electrospinning time impacted nanofiber distribution. The 0.1mm + 8%(16 min) and 0.1mm + 16%(16min) had a greater amount of fibers present than the 0.1mm + 8%(8 min) and 0.1mm + 16%(8 min). A greater amount of fibers covers a greater area of the scaffold so there is more potential to guide more neurites which is why the 0.1mm + 8%(16 min) and 0.1mm + 16%(16min) groups showed better neurite extension. The 0.1mm + 8%(8 min) and 0.1mm + 16%(8 min) inner fiber coverage parallels the behavior of the 0.1mm group and the tubular group as there is a significant area of the scaffold without fibers which limits the amount of neurite that can be guided and in turn may influence how many neurite can extend from the PC12 cells.
- We incorporated Laminin, an ECM protein, in the nanofibers in an aligned nanofiber orientation within our scaffold through the use of PEG to cross link PCL, thus preventing the protein desorption once the scaffold is implanted. We assessed the effectiveness of PEG in crosslinking using PC12 cells. Based on the neurite extension and neurite density of the PC12 cells, we deduced concentrations 2 mg of Laminin and 4 mg of Laminin are optimal for incorporation within nanofibers.
- The mechanical tests of PCL-gelatin and PCL-sodium alginate were improved. Gelatin, modified gelatin, sodium alginate degraded and sodium alginate non-degraded were tested for biocompatibility using various cells.
- Gelatin, modified gelatin, sodium alginate degraded and non-degraded were compatible to various cells. The cell proliferations were evident for these polymers.
- Cell adhesion, compatibility and conductivity studies using sulfonated polymers of poly(methyl vinyl ether-alt-maleic anhydride) (PMVEMA), poly(ether ether ketone) (PEEK), poly(ether sulfone) (PES) and poly(phenylene oxide) (PPO) indicated that these polymers could be useful for nerve regeneration applications.

5. **CONCLUSION:**

Since initiating this project, we have observed that in the fabrication of our spiral scaffold that a 0.1 mm wall thickness is more desirable than a 0.2 mm wall thickness as it can allow more axon infiltration and yield more layers for the spiral structured scaffold thus allowing a greater surface area for nerve regeneration. Additionally we have observed that 0.1mm + 16%(16min) yields the nanofiber diameter and nanofiber distribution which provides a more favorable environment for neurite extension and neurite density. We have also assessed that PEG is capable of crosslinking PCL to allow better incorporation of ECM proteins, such as Laminin. The results obtained from biocompatibility studies conclude that these materials could be implantable. Also the neurite extension in presence of NGF indicates that the nanofibers could be useful for nerve regeneration applications. Also the conduction polymers could have potential for applications in nerve regeneration. As we initiate activities for the second year, we will focus on incorporating growth factors into our scaffold as well as assessing the optimal concentrations and bioactivity. Upon completion of our preliminary studies, our work will culminate into studies in the rat sciatic nerve model and assessing the effectiveness of

our spiral scaffold for a 15 mm gap length, which is equivalent to the critical gap length of repair in humans, 40 mm.

By optimizing the variables that influence nerve graft fabrication as well as optimizing the additional factors that can be incorporated in nerve grafts to aid nerve regeneration we can ideally develop a nerve graft that can operate at optimal efficiency to aid and induce nerve regeneration for critical gap lengths. By successfully treating critical gap lengths, our project will be applicable for all gap lengths of peripheral nerve injury. As aforementioned military personnel are more prone to peripheral nerve injuries, often due to the nature of their work, can our project can alleviate some of the challenges faced by the men and women in uniform and ideally trickle down to civilian application.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Book Chapter
Ramos, D.M., Peach, M.S., Yu, X., Mazzocca, A.D., Kumbar, S.G., "Tendon Tissue Engineering" in "Engineering Musculoskeletal Tissues and Interfaces" Edited by Nukavarapu, Freeman and Laurencin 2014, Woodhead Publishing Limited (Accepted-In press)

Research Articles
Jaiswal D., James, R., Shelke N.B., Harmon M.D., Brown J. L., Hussain F., and Kumbar, S.G., Gelatin Nanofiber Matrices Derived from Schiff Base Derivative for Tissue Engineering Applications. *J. Biomed. Nanotechnol.,* 2014 (Communicated)

James, R., Nagarale, R.K., Sachan, V., Badalucco, C., Bhattacharya P., and Kumbar, S.G., Synthesis and Characterization of Electrically Conducting Polymers for Regenerative Engineering Applications: Sulfonated Ionic Membranes. *Polym. Adv. Tech.,* 2014, DOI: 10.1002/pat.3385

Nada, A.A., James, R., Shelke, N.B., Harmon, M.D., Awad, H. M., Nagarale, R. K., Kumbar, S.G., Smart Methodology to Fabricate Electrospun Chitosan Nanofiber Matrices for Regenerative Engineering Applications. *Polym. Adv. Tech.,* 25 (5), 507-515, 2014.

Shelke, N.B., James, R., Laurencin, C.T., Kumbar, S.G., Polysaccharide Biomaterials for Drug Delivery and Regenerative Engineering. *Polym. Adv. Tech.,* 25 (5), 448-460, 2014.

Conference Proceedings/Abstracts
Wei Chang, Munish Shah, Kathleen McGuire, Sangamesh G Kumbar, Cato Laurencin, Xiaojun Yu. "Development of A Novel Structured Nanofibrous Nerve Guidance Conduit for Nerve Regeneration" Global Biotechnology Congress 2014 held in Boston, MA from June 15 to June 19 of 2014.

Alejandra, M., Ramos, D.M., Laurencin, C.T., Kumbar, S.G., "Peptide linkage of Poly(caprolactone)-Chitosan Blend Scaffolds" BMES 2014 Annual Meeting, San Antonio, Texas, October 22-25, 2014 Poster #3118

Ramos, D.M., Laurencin, C.T., Kumbar, S.G., "Tendon Differentiation Using Human Recombinant Insulin" BMES 2014 Annual Meeting, San Antonio, Texas, October 22-25, 2014 Poster # 653

Aravamudhan, A., Ramos, D.M., Harmon, M.D., Kumbar, S.G., "Characterization of Polysaccharide Based Micro-Nano Structured Scaffolds for Osteoinductivity" BMES 2014 Annual Meeting, San Antonio, Texas,October 22-25, 2014 Poster # 492

Kumbar, S.G., "Micro-Nano Structured Bioactive Fiber Matrices for Soft Tissue Regeneration" The New England Musculoskeletal Institute (NEMSI)-Research Day on April 4, 2014 at at UCONN Health Center (Podium)

Kumbar, S.G., "Polysaccharide Biomaterials for Drug Delivery and Regenerative Engineering Applications" National Wind Resource Center, Department of Mechanical Engineering Department- Texas Tech University, June 13, 2014 (Podium)

7. INVENTIONS, PATENTS AND LICENSES:

Patent filed:
Xiaojun Yu, Wei Chang. "Implantable nerve conduit having a polymer fiber spiral guidance channel". US utility patent No. 14/313,384 filed on June 24, 2014.

8. REPORTABLE OUTCOMES:
   a. 0.1 mm Spiral Nerve Guidance Conduit coated with inner aligned fibers wrapped with an outer tubular structure
   b. 0.2 mm Spiral Nerve Guidance Conduit with inner aligned fibers wrapped with an outer tubular structure

9. OTHER ACHIEVEMENTS: Nothing to report.

10. REFERENCES: List all references pertinent to the report using a standard journal format
[1] Yang, Yumin, et al. "Repair of rat sciatic nerve gap by a silk fibroin-based scaffold added with bone marrow mesenchymal stem cells." Tissue Engineering Part A 17.17-18 (2011): 2231-2244.
[2] Hu, Xueyu, et al. "A novel scaffold with longitudinally oriented microchannels promotes peripheral nerve regeneration." Tissue Engineering Part A 15.11 (2009): 3297-3308.
[3] Zhang, Yong-Guang, et al. "Omentum-wrapped scaffold with longitudinally oriented micro-channels promotes axonal regeneration and motor functional recovery in rats." PloS one 6.12 (2011): e29184

11. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

None.